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Novel small molecule inhibitors of Jak2/STAT3 pathway: design, structure-activity relationship, and oral bioavailability

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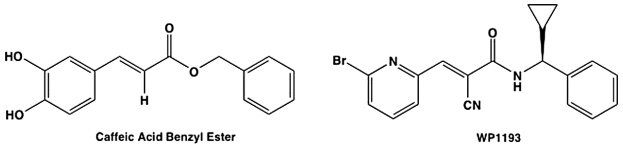
Signal transducer and activator of transcription-3 (STAT3) is constitutively activated in many types of cancer, including pancreatic carcinoma, glioblastoma multiforme, and squamous cell carcinoma of the head and neck. STAT3 activation can promote tumor cell proliferation and survival as well as VEGF expression, angiogenesis, and metastasis in vivo. Because EGF- and IL-6 receptor–mediated signaling induces phosphorylation of Jak2 and then STAT3, both proteins seem excellent targets for drug development. Currently available inhibitors of Jak2/STAT3 activation (e.g., AG490) can inhibit Jak2/STAT3 phosphorylation but only at 50–100 μM concentrations and thus are not active in vivo. Because AG490 shows striking structural similarity to the natural products of the caffeic acid family, we compared the in vitro activity of caffeic acid benzyl ester to AG490 in the Colo357 cell line. Both compounds demonstrated similar activity, which led us to design and synthesize novel inhibitors with far greater potency. This new class of compounds inhibits Jak2 and STAT3 phosphorylation and the related downstream transcription of pro-survival proteins, thereby inhibiting tumor growth in vitro and in vivo. For these studies, all analogs were tested against Colo357-FG pancreatic and U87-MG glioblastoma multiforme cancer cell lines. The most potent compounds were shown to inhibit not only constitutively activated Jak2/STAT3 but also IL-6–stimulated STAT3 activation, suppressing the expression of Stat3 upregulated proteins c-myc, Bcl-2, Bcl-X_L, survivin, and Mcl-1 and inducing apoptosis, all at low micromolar concentrations. To select analogs suitable for more comprehensive preclinical evaluations, we evaluated the pharmaceutical characteristics of the most potent analogs and identified WP1193 as a compound displaying excellent activity and also good oral bioavailability. Preclinical examinations of the in vivo activity, drug biodistribution, and metabolism of WP1193 are currently underway.



Caffeic Acid Benzyl Ester WP1193

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Effect of surgical tumor resection methods on expression of signaling molecules in mouse models

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Surgical stress is thought to have an effect on the mortality and residual tumor progression after surgery; therefore diminishing surgical stress is important. Effect of the surgical stress on the signaling molecules has not been explored. The purpose of this study was to assess in a murine model whether the location of tumor or the resection method has an effect on signalling molecules such as pERK (MAP kinase pathway), pAKT (AKT pathway) or pp38 (MEK pathway). We have evaluated the levels of signaling molecules in subcutaneous and orthotopic MCF-7 human breast tumor xenografts in nude mice as well as in hollow fibers implanted intraperitoneally (IP) and subcutaneously (SC) in these mice. We also compared several methods of tumor tissue collection using cryobiopsy, needle aspiration and classical resection of Colo 829 melanoma tumors implanted subcutaneously into nude mice. The levels of phosphoproteins and total proteins from these tumors were evaluated using western blot analysis. We observed an increase in pERK and total ERK as well as PARP in orthotopic tumors as compared to SC tumors in MCF-7 tumors implanted in nude mice. Quantitatively the levels of pERK, pAKT and pp38 were higher in hollow fibers implanted IP compared to fibers implanted SC. A significant difference was observed in the expression of phosphoproteins and other signaling molecules in tumor tissues collected by cryobiopsy compared to standard resection. In conclusion, the resection method, tumor growth location as well tumor model can affect the signaling molecules. Therefore, one must be cautious during development of experimental protocols to assure that the selected models and methods optimally support the desired experimental goals.

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Epithelial mesenchymal transition (EMT) and hMena expression as determinants of sensitivity of pancreatic adenocarcinoma (PDAC) cell lines to EGFR tyrosine kinase inhibitors (TKI)

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Erlotinib (Tarceva™) is an ATP-competitive EGFR (TKI), that has demonstrated to modestly, but significantly, extend survival in advanced PDAC patients. Here, we sought to identify biomarkers that differentiate erlotinib-responsive from non-responsive PDAC cells. Nine wild-type EGFR human PDAC cell lines were characterized for the expression of EMT markers (E-cadherin, N-cadherin, and vimentin) and hMena, a member of the Ena/VASP family of proteins, involved in the regulation of cell motility and adhesion through actin cytoskeleton remodeling. The growth-inhibitory activity of erlotinib *in vitro*, measured by MTT assay, varied widely among the different cell lines: the four cell lines expressing the epithelial marker E-cadherin (L3.6pl, BxPC3, T3M4, PACA44) showed striking sensitivity (IC_{50}: 0.1–1 μM), while the four cell lines (MiaPaca2, Panc1, HS766T, PT45) expressing N-cadherin and/or vimentin (phenotypic hallmarks of EMT) and the cell line with concurrent expression of epithelial and mesenchymal markers (CfPac1) were relatively resistant (IC_{50} > 10 μM). Interestingly, 2/4 E-cadherin positive and N-cadherin and/or vimentin negative cell lines showed an high level of TGF-α production and constitutive EGFR phosphorylation, whereas a modest TGF-α production and no constitutive EGFR phoshorylation was observed in the N-cadherin and/or vimentin-expressing, erlotinib-resistant cell lines, suggesting the presence of an autocrine, EGFR-mediated, signalling loop in cells with an epithelial phenotype. The analysis of the expression of hMena and its splice variants by western blot and RT-PCR demonstrated a phenotypic correlation between EMT markers, erlotinib sensitivity, and a differential hMena isoform expression pattern. Preliminary data also suggest that the phenotype correlates with functional hMena activity. In fact hMena knockdown with a human ENA-specific siRNA construct reduced the *in vitro* growth rate of the mesenchymal Panc1 cell line as compared to a non-specific siRNA construct. Overall, our data suggest that the EMT status and the differential expression of hMena isoforms are closely related in preclinical models of PDAC and might mark the transition from an epithelial, EGFR signalling-dependent, to a mesenchymal, TKI-resistant, phenotype. These findings may have important implications for the development of novel, molecularly-targeted approaches for the treatment of PDAC.

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AV-412, a potent EGFR/HER2 TK inhibitor causes tumor regression in novel genetically engineered EGFRL858R and EGFRL858R&T790M lung tumor models

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Background: AV-412 (formerly known as MP-412) is a potent and selective inhibitor of the tyrosine kinase (TK) activities of the receptor for epidermal growth factor (EGFR) and HER2 that exerts potent tumor inhibitory activity against wild type and mutant forms of EGFR-TK. AV-412 is scheduled to enter clinical development in 2006.
Material and Methods: AVEO's platform for making complex inducible tumor models uses genetically modified ES cells to generate tumor bearing chimeric mice. Using this platform, multiple lung adenocarcinoma models driven by EGFR mutants, Kras, and HER2 genes known to be altered in human lung cancers have been generated. Furthermore, tumors originally driven by inducible expression of HER2 or Kras have been functionally complemented *in vivo* by expression constructs of EGFR mutants, which provided precise control of the genetic context of the tumor. Tumors from these models have been propagated *in vivo* and tumor inhibitory activity of AV-412 has been examined.
Results: AV-412 is highly active against tumors from the chimeric model carrying $EGFR^{L858R}$ mutation. One mg/kg daily dosing, which is 1/100th of

maximum tolerated dose in mice resulted in significant tumor regression. Erlotinib is also active in these tumors, which is consistent with clinical findings. In contrast, engineered lung tumors driven by Kras do not exhibit regression even at much higher dosing levels. More importantly, AV-412 is active against engineered tumors driven by the drug resistant mutant $EGFR^{L858R\&T790M}$ derived by *in vivo* functional complementation. In addition, AV-412 is also active against chimeric breast tumors driven by HER2.
Conclusion: These results provide direct evidence in a genetically defined system for determining the specific genetic context of AV-412 response, which in turn provide insights for AV-412 clinical activity and is strongly suggestive of the utility of the platform for human drug response prediction.

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Inhibition of erbB1/2 by small molecule tyrosine kinase inhibitors, but not trastuzumab, affects metabolic pathways: implications to cardiac toxicity

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Background: Therapies targeting ErbB2 represent an attractive strategy in breast cancer. Trastuzumab, an anti-ErbB2 monoclonal antibody, is an approved treatment for patients with ErbB2-overexpressing breast cancers. Tykerb is a potent, reversible inhibitor of ErbB2 and ErbB1 tyrosine kinase (TKI) and is currently in Phase III clinical trials in breast and other carcinomas. The principal adverse event attributable to trastuzumab is cardiac toxicity. This study was conducted to elucidate mechanisms that affect metabolic pathways by a TKI and an antibody directed to ErbB2 and their effects on breast cancer cells, primary human adipocytes and cardiomyocytes.
Material and Methods: Western blotting was used for pAkt, pErk1/2, pAMPKα and peEF2 (Cell Signaling, Beverly, MA); ERRα, ERRγ (R&D Systems Minneapolis, MN); PGC-1 (Chemicon International, Temecula, CA); MCAD (Cayman Chemicals, Ann Arbor, MI) and Actin (Sigma, St. Louis, MO). Lipid staining: cells were fixed in NBF and stained with Oil Red O (Sigma). Cells: AU565, breast cancer cells, primary cardiomyocytes, and adipocytes were grown in RPMI supplemented with 15% BFS and treated with BAPTA/AM (Calbiochem): 5–30 μM; GW-2974 (Sigma): 1–25 μM; trastuzumab (Genentech): 5–50 mg/ml; Heregulin (LabVision, Fremont, CA): 5–100 ng/ml.
Results: Our results show that treatment with GW-2974 (or with Tykerb) directed to ErbB1/2 alter fatty acid metabolic pathways through activation of adenosine monophosphate kinase (AMPK), a key regulator in mitochondrial energy producing pathways in human cardiac cells, adipocytes and breast cancer cells. The changes include phosphorylation of AMPK and eEF2, upregulation of ERRα and PGC-1, activators of fatty acid oxidation, in cardiomyocytes, and downregulation of lipid expression in human cardiomyocytes, adipocytes, breast cancer cells, as well as downregulation of fatty acid synthase (FAS), increased lipid oxidation and changes in ion and calcium channels. The metabolic changes were reversed by calcium chelation. Trastuzumab, which downregulated FAS and changed ion channels, failed to activate AMPK, but downregulated survival pathways such as AKT and Heregulin.
Conclusions: Our results show that treatment using TKI to inhibit ErbB1/ErbB2 pathways in breast cancer cells, in human cardiomyocytes, and adipocytes results in activation of AMPK through changes in calcium channels. AMPK regulates cellular energy homeostasis. Activation of AMPK after stress is associated with protection of cells against injury such as ischemia and nutrient depletion, thereby helping to preserve the levels of cellular ATP. Thus, activation of AMPK will likely provide protection against cellular damage by ErbB targeted therapy alone or when given in combination with chemotherapy. The failure of trastuzumab to activate AMPK together with downregulation of survival pathways may point to the source of its cardiac toxicity.

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Discovery of a novel FAK inhibitor, NVP-TAE226, and its activities on in vivo and in vitro models

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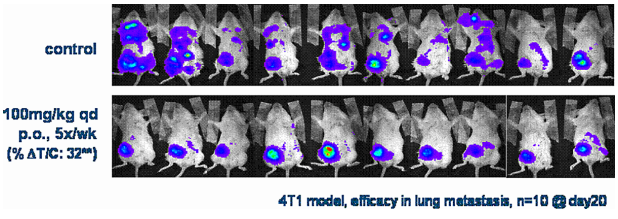
Background: Focal Adhesion Kinase (FAK) is an attractive anti-cancer drug targets because FAK is a key molecule of tumor cell proliferation, migration, and survival. FAK is generally overexpressed in various types of tumor cells and is closely correlated with invasive potential. FAK levels are greatest in highly metastatic tumors. Activation of integrins and the growth factor receptors result in FAK autophosphorylation at Y397 and the presentation of suitable binding sites for proteins containing either SH2 or phosphotyrosine binding domains. Recent evidences indicate that FAK plays important roles in cancer cell proliferation and survival. A selective FAK inhibitor would be expected to halt or kill invasive tumor cells, and potentially interfere with normal cell migration (e.g. endothelial cells).
Methods: We have discovered NVP-TAE226, a novel small molecule inhibitor of FAK. The compound was evaluated in kinase enzymatic assays, cell-based kinase assays and *in vivo* models. Anti-metastasis effect was evaluated by applying *in vivo* imaging. All procedures in this study were in compliance with the regulations of Animal Welfare Committee in Novartis Institutes for BioMedical Research Tsukuba.
Results: NVP-TAE226 inhibits FAK with low nanomolar IC50 values in a purified kinase enzymatic assay. In cell-based kinase assays, FAK was inhibited with an IC50 range of 100 to 300 nM compared to the other kinases tested which were >10-fold less sensitive. Oral administration of NVP-TAE226 showed potent inhibition of orthotopic tumor growth and spontaneous metastasis in a dose-dependent manner. The compound was well tolerated in mice in terms of body weight changes. Inhibition of FAK autophosphorylation at Y397 and Akt phosphorylation at Serine473 was observed in a dose-dependent manner in 4T1 breast carcinoma.



Conclusion: NVP-TAE226 represents a novel class of selective and small molecule kinase inhibitors that have potential clinical applications with a potent *in vivo* activity.

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Inhibition of MEK1/2 signalling with CI-1040 in human melanoma cells leads to alterations in phosphocholine metabolism

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Background: RAS-RAF-MEK-ERK (or MAPK) signaling is deregulated in many cancers, especially melanomas, and inhibitors of this pathway are now in clinical trials. Detecting biomarkers of MAPK signaling inhibition could facilitate the clinical evaluation of this novel therapy. Using magnetic resonance spectroscopy (MRS), we have previously shown that treatment with the early prototype MEK inhibitor U0126 correlated with a drop in phosphocholine (PC) levels in human breast and colon cancer cells (1). Here we investigate: a) whether inhibition with the MEK1/2 selective inhibitor CI-1040 in human melanoma cells could trigger similar metabolic effects as U0126; and (b) the mechanistic basis for any observed changes.
Materials and Methods: WM266.4 human malignant melanoma cells were treated with 0.2 μM, 0.5 μM or 1 μM CI-1040 for 24h or with 1 μM CI-1040 for 3h, 6h, 16h and 24h. For mechanistic studies, cells were also treated with 1 μM CI-1040 for 24h followed by a 3h incubation in fresh medium containing 1 μM CI-1040 and 100% 100 μM [1, 2]-^{13}C-choline. Inhibitor action was verified by Western blotting for P-ERK1/2 and cyclin D1 levels.